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Crystallization and preliminary X-ray analysis of adenylylsulfate reductase from *Archaeoglobus fulgidus*

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A group of anaerobic microorganisms use sulfate as the terminal electron acceptor for energy conservation. The process of sulfate reduction involves several enzymatic steps. One of them is the conversion of adenylyl sulfate (adenosine-5′-phosphosulfate) to sulfite, catalyzed by adenylylsulfate reductase. This enzyme is composed of a FAD-containing α-subunit and a β-subunit harbouring two [4Fe–4S] clusters. Adenylylsulfate reductase was isolated from *Archaeoglobus fulgidus* under anaerobic conditions and crystallized using the hanging-drop vapour-diffusion method using PEG 4000 as precipitant. The crystals grew in space group $P2_12_12_1$, with unit-cell parameters a = 72.4, b = 113.2, c = 194.0 Å. The asymmetric unit probably contains two $\alpha\beta$ units. The crystals diffract beyond 2 Å resolution and are suitable for X-ray structure analysis.

1. Introduction

Sulfate is used as terminal electron acceptor for energy conservation by a group of anaerobic microorganisms which can enzymatically reduce sulfate to sulfide (Thauer *et al.*, 1977). This process, designated as dissimilatory sulfate reduction, is basically performed by four enzymes: ATP-sulfurylase, inorganic pyrophosphatase, adenosine-5′-phosphosulfate reductase (APS reductase; E.C. 1.8.99.2) and sulfite reductase. Prior to reduction, the inert sulfate has to be activated to adenosine 5′-phosphosulfate (APS) at the expense of ATP *via* ATP sulfurylase. APS is subsequently reduced by APS reductase to AMP and sulfite, the latter being further reduced to sulfide by sulfite reductase (Steuber *et al.*, 1995; Hansen, 1994; Peck & LeGall, 1994).

Sulfate-reducing microorganisms play an important role in the biogeochemical sulfur cycle on earth and inhabit aquatic and terrestrial sediments. They belong mostly to the domain of bacteria, except for *Archaeoglobus* which is an archaeal organism. *A. fulgidus* is a hyperthermophilic organism mainly found in the ocean and in terrestrial oil deposits (Stetter *et al.*, 1987; Stetter, 1988).

APS reductase is located in the cytoplasm of several sulfate-reducing and sulfur-oxidizing organisms (Fritz *et al.*, 2000). The enzyme is a heterodimeric complex composed of an α-subunit with a molecular mass of 70–75 kDa and a β-subunit of 18–23 kDa (Fritz *et al.*, 2000). Analysis of the primary structure of APS reductase (Fritz *et al.*, 2000; Speich *et al.*, 1994) and spectroscopic investigations revealed that the α-subunit contains one FAD and the β-subunit contains two [4Fe–4S] clusters with largely differing redox potentials (Fritz *et al.*, 2000; Lampreia *et al.*, 1994). From magnetic interactions between the two clusters, a mutual distance less than 15 Å was proposed (Fritz *et al.*, 2000).

The reaction mechanism of APS reductase has not yet been determined. Two alternative catalytic mechanisms were proposed. One mechanism is based upon an intermediate between a cysteine sulfhydryl group and sulfite (Peck *et al.*, 1965), whilst the second is based upon a FAD–sulfite intermediate (Michaels *et al.*, 1970).

A three-dimensional structure would help in understanding this interesting biochemical reaction at atomic level as well as in the analysis of the surroundings of the cofactors and the electron-transfer pathway. Therefore, we initiated an X-ray structure analysis and report here on the isolation, crystallization and preliminary crystallographic analysis of APS reductase from *A. fulgidus*.

2. Materials and methods

2.1. Cell growth and purification

A. fulgidus DSM 4304T was grown as described previously (Stetter *et al.*, 1987). Purification of APS reductase was performed under strict exclusion of oxygen. All chromatographic steps were carried out with an Amersham Pharmacia FPLC system in a Coy anaerobe chamber (95% N_2, 5% H_2). Typically, 15 g cells (wet weight; 0.5–0.6 g ml^{-1}) were

suspended in 10 m*M* potassium phosphate pH 7.0 (buffer *A*) containing a few crystals of DNAse and 1 m*M* $MgCl_2$. Cells were disrupted by one passage through a French press cell (138 MPa). Crude extracts were separated into soluble and membrane fractions by ultracentrifugation (100 000*g* for 1 h). The soluble fraction containing APS reductase was applied to a Q-Sepharose fast-flow column (5.0 × 10 cm) equilibrated with buffer *A*. APS reductase was eluted at about 0.10 *M* KCl using a linear gradient (0–1.0 *M*) of KCl. Pooled APS reductase fractions were dialyzed against buffer *A* or concentrated in a ultrafiltration stirring cell before diluting with buffer *A*. After loading onto a ResourceQ30 column (1.6 × 12 cm) equilibrated with 20 m*M* potassium phosphate and 5%(*v*/*v*) glycerol pH 7.0, APS reductase was eluted at 0.15 *M* KCl after applying a linear gradient of 0–0.5 *M* KCl. The enzyme was then concentrated by ultrafiltration (30 kDa cutoff; Amicon) and loaded onto a Superdex 200 gel-filtration column (2.6 × 60 cm) which was equilibrated with 50 m*M* potassium phosphate, 150 m*M* KCl, 5%(*v*/*v*) glycerol pH 7.0. APS reductase fractions, which still contained minor impurities, were diluted and further purification was achieved by chromatography on a ResourceQ15 column (1.0 × 12 cm). After elution with a linear KCl gradient, the pure protein was dialyzed against 10 m*M* Tris–HCl pH 7.6 and concentrated to 15–20 mg ml^{-1}. The protein concentration was determined by the Microbioret method (Goa, 1953) after trichloroacetic acid precipitation (Bensadoun & Weinstein, 1976). Finally, protein aliquots of 100 µl were frozen and stored in liquid nitrogen prior to crystallization.

2.2. Analytic methods

The purity of the APS reductase was analyzed by SDS–PAGE carried out with the Protean II electrophoresis system (220 × 200 × 0.75 mm; BioRad) or with the Mighty Small System (100 × 80 × 0.75 mm; Hoefer Scientific Instruments) using 12.5% acrylamide gels (Laemmli, 1970). Gels were stained with Coomassie blue (Zehr *et al.*, 1989) or with silver (Rabilloud, 1990).

The structural and functional integrity of the protein was analyzed by the characteristic UV–Vis absorption and EPR spectroscopic properties of the cofactors.

The activity of APS reductase was determined according to a modified procedure of Peck *et al.* (1965). The reaction mixture in a rubber-sealed cuvette contained 2 m*M* sodium sulfite, 2 m*M* AMP, 0.5 m*M* EDTA, 1 m*M* potassium ferricyanide in 50 m*M* Tris–HCl pH 7.6. The reaction was started by addition of enzyme through a gas-tight syringe. The activity was measured at 353 K as micromoles of APS formed per minute using the absorption decrease at 420 nm for ferricyanide (ε_{420} = 1020 M^{-1} cm^{-1}).

2.3. Crystallization and data collection

The enzyme was crystallized using the hanging-drop vapour-diffusion method performed in an anaerobic chamber (95% N_2, 5% H_2). An initial screening at 277 K using the commercial Hampton Research crystallization kit I (Jancarik & Kim, 1991) revealed microcrystals using several PEG agents as precipitant. The most suitable crystals were obtained when using reservoir conditions of 4–6% PEG 4000, 0.1 *M* NaAc pH 4.8 and 0.2 *M* NaCl. The drop solution was composed of equal amounts of protein and reservoir solution.

X-ray diffraction experiments were performed in-house with a Rigaku RU-200 X-ray generator using Cu $K\alpha$ radiation and a MAR Research imaging-plate detector. Native data were collected at the Max-Planck beamline BW6 at the Deutsches Elektronensynchrotron in Hamburg using a MAR CCD detector. All measurements were achieved under flash-freezing conditions after soaking the crystals in a cryoprotectant solution containing 6% PEG 4000, 0.1 *M* NaCl, 0.1 *M* NaAc pH 4.8 and 25% glycerol. Measured reflection intensities were processed with *DENZO* and scaled with *SCALEPACK* (Otwinowski, 1993).

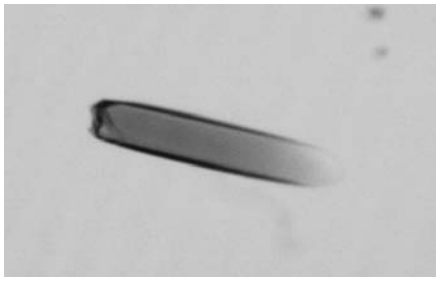

Figure 1
Crystal of APS reductase. The dimensions of this yellow–brownish crystal are 200 × 300 × 600 µm.

3. Results and discussion

3.1. Purification and characterization

APS reductase from *A. fulgidus* was isolated for the first time under the exclusion of oxygen. The enzyme was purified by a new procedure including three chromatographic steps performed at room temperature in the anaerobic chamber. In order to minimize protein denaturation by thermally induced unfolding or by protease activity, the complete purification was performed within 36–48 h. The yield of pure APS reductase was very high (50 mg per 10 g cells wet weight) and only two bands (subunits α and β) were visible on the SDS–PAGE gel.

The structural integrity and enzymatic activity was verified by spectroscopic and kinetic methods. The UV–Vis absorption spectrum was very similar to that of APS reductases from other organisms such as *Desulfovibrio* sp. or *Thiobacillus denitrificans* (Fritz *et al.*, 2000) as indicated by the characteristic absorption maxima at 278 and 388 nm for flavin and iron–sulfur centres, respectively. Analysis of the cofactors revealed the presence of one FAD and two [4Fe–4S] clusters per $\alpha\beta$ unit (Fritz *et al.*, 2000). According to EPR spectroscopic measurements both [4Fe–4S] clusters were intact. This result is in contrast to previous observations (Lampreia *et al.*, 1994), where signals of a [3Fe–4S] cluster were detected. This cluster is most likely a product of oxidative degradation of a [4Fe–4S] cluster. Storage of the enzyme in the presence of oxygen at 277 K led to a minor decrease in absorption between 300 and 500 nm after 4 d, most probably owing to oxidation of [4Fe–4S] clusters, whereas protein stored under exclusion of oxygen showed no spectral changes. The pure protein had an activity of 1.2 µmol min^{-1} mg^{-1}, which corresponds well to previous results (Speich *et al.*, 1994). One cycle of flash-freezing in liquid nitrogen and thawing affected neither the activity nor the spectral properties of the enzyme.

3.2. Crystallization and data collection

The crystallization of the functionally intact enzyme was performed under anaerobic conditions at a temperature of 277 K. Yellow–brownish coloured rod-shaped crystals with dimensions of 0.2 × 0.4 × 0.7 mm appeared within 2–3 d (Fig. 1). The crystals belonged to the space group $P2_12_12_1$, with unit-cell parameters a = 72.4, b = 113.2, c = 194.0 Å. The packing densities V_M of 4.2 and 2.1 Å^3 Da^{-1} are compatible with one or two heterodimers per asymmetric unit, respectively. The derived solvent contents were calculated to be 70 and 40%, respectively, which are both in the range for water-soluble proteins (Matthews, 1968). Self-rotation calculations, however, supported the presence of two heterodimers

Table 1
Data-collection statistics for an APS reductase crystal.

Values in parentheses refer to the highest resolution shell.

Resolution (Å)	2.5–30.0 (2.5–2.54)
Completeness (%)	96.5 (89.1)
Redundancy	3.1 (1.8)
$I/\sigma(I)$	25.9 (14.3)
R_{sym} (%)	3.3 (4.9)

per asymmetric unit. The crystals diffracted to beyond 2 Å resolution.

A native data set has been collected at the Max-Planck beamline BW6 to 2.5 Å resolution at a wavelength of 1.06 Å. 165 042 reflections were measured and reduced to 54 098 unique reflections, which corresponds to a completeness of 96.5% in the resolution range 30.0–2.5 Å. The R_{sym} value was determined to be 3.3% in this range (Table 1).

Phase determination by the multiple anomalous dispersion method using the eight Fe atoms of the two [4Fe–4S] clusters is in progress.

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